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CLAIMS

[Claim(s)]
[Claim 1]A transgenic plant into which a gene which encodes fused protein of a membrane immunity carrier and Japanese encephalitis virus outline protein was introduced.
[Claim 2]The transgenic plant according to claim 1 in which said membrane immunity carrier is cholera toxin B chain protein (CTB).
[Claim 3]The transgenic plant according to claim 1 or 2 in which said Japanese encephalitis virus outline protein is Eglycoprotein.
[Claim 4]A transgenic plant into which pBI121-CTB-JE was introduced.
[Claim 5]The transgenic plant according to any one of claims 1 to 4 in which said vegetation is tobacco.
[Claim 6]The transgenic plant according to any one of claims 1 to 5 for using it as a Japanese encephalitis virus vaccine. .

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention relates to the transgenic plant into which the gene which encodes the fused protein of a membrane immunity carrier and Japanese encephalitis virus outline protein (E glycoprotein) was introduced.
[0002]
[Description of the Prior Art]The conventional vaccine containing background Japanese encephalitis vaccine of an invention has a killed vaccine in use, and the inoculation using an injector is most widely used about the vaccine law. When a virus was inoculated in the brain of a mouse and a virus increased, the present Japanese encephalitis vaccine takes out a brain and performs separation, refining, and processing of inactivation for a virus. Such a vaccine has a still more serious cost problem, although there is also an ethical problem of sacrificing many animals. Although the spread of the vaccines of a conventional type is satisfactory in any way in advanced nations, the economic developing country of Asia, Africa, and the countries in Central America of being afflicted by spread of many infectious diseases, such as malaria, dengue, and Japanese encephalitis, is almost the case.
[0003]Since it says that expense starts the spread too much, the actual condition is it being very difficult to spread inoculation of existing vaccines in Southeast Asia or an African area, and being unable to perform smoothly the precautions against infectious disease in a worldwide scale about the group vaccination in an economic developing country.
[0004]Permucosal immunization made by gene modification technology, such as taking orally of a recombination protein vaccine and pernasality, is ****** type vaccines which do not need an injector. Existing vaccines are excelled also in cost.
It is possible to acquire an equivalent effect also immunologically. "The vaccine to eat" which uses recombination vegetation also especially in it is the ultimate vaccine law of a permucosal vaccine, and is extremely excellent in the cost aspect. If the spread of vaccines takes into consideration the global situation of being very difficult, from an economical problem, the future vaccine development needs to perform preponderantly various permucosal vaccines containing vegetable origin recombination vaccines, such as a vaccine to eat. It is a field attractive [in advanced nations] also from an international-contributions position also as new industry.
[0005]The substance which functions as a conventional technology Japanese encephalitis virus vaccine is protein. Japanese encephalitis vaccine is a killed vaccine refined from the infection mouse brain emulsion,

and is inoculated by subcutaneous injection. At the Japanese encephalitis which the route of infection spreads like polio or influenza not by the membrane of an intestinal tract or a breather but by sucking blood of a medium mosquito, there was no way of thinking of inoculating a vaccine from membrane. However, if a nasal cavity etc. to membrane immunity is possible, it is expected that an injector will become insoluble and will lower inoculation cost substantially. Then, to inoculate Japanese encephalitis vaccine from nasal cavity membrane was tried. As a result, the nasal cavity inscribed kind method can derive the same virus infection defense effect as the conventional vaccine law. In order to derive an antibody specifically to the Japanese encephalitis virus particles by mucosal administration, The fusion gene of the cholera toxin B chain (CTB) and Japanese encephalitis virus outline protein (JE) (E glycoprotein) which have specific compatibility in a permucosal immunity organization is produced, By introducing the gene into each living thing kind (bacteria, yeast), production of fused protein (CTB-JE E glycoprotein) was enabled. The fused protein of CTB-JE Eglycoprotein available as the above-mentioned Japanese encephalitis virus vaccine is produced in various gene expression systems, such as bacteria, yeast, an insect cell. It was possible by extracting fused protein from those expression systems, and prescribing a medicine for the patient from taking orally and pernasal membrane to have derived the phylaxis immunity of a whole body system.

[0006]When were conventionally inoculated by subcutaneous injection, and impurity was contained, the immune response to the impurity may have been derived, but. When permucosal administration was carried out by internal use, the immune response by impurity was able to derive the immune response only to the protein by which absorption combination is carried out via membrane, without being derived.

[0007]It is possible to carry out immunity via a direct intestinal mucosa by carrying out gene expression to edible vegetation especially in the case of plant vaccine. The above-mentioned composition and its membrane immunity prescribing [for the patient]-a medicine method are applicable to a vertebrate at large [containing Homo sapiens and livestock animals].

The use as "a vaccine to eat" is expected.

Then, production of the transgenic plant into which the above-mentioned gene was introduced was desired as permucosal Japanese encephalitis vaccine.

[0008]

[Problem(s) to be Solved by the Invention]In order to solve such a problem, an object of this invention is to provide the transgenic plant into which the gene which encodes Japanese encephalitis vaccine (fused protein of a membrane immunity carrier and Japanese encephalitis virus outline protein (E glycoprotein)) was introduced.

[0009]

[Means for Solving the Problem]In order that this invention persons may solve an aforementioned problem, as a result of inquiring wholeheartedly, fused protein of a membrane immunity carrier (cholera toxin B chain protein (CTB)) and Japanese encephalitis virus outline protein (E glycoprotein), It succeeds in producing a transgenic plant which finds out functioning as Japanese encephalitis vaccine, and reveals said protein, and came to complete this invention.

[0010]That is, this invention provides a transgenic plant containing a gene which encodes fused protein of a membrane immunity carrier and Japanese encephalitis virus outline protein (Eglycoprotein).

[0011]This invention provides said transgenic plant and the transgenic plant according to claim 1 or 2 in which said Japanese encephalitis virus outline protein is Eglycoprotein in which said membrane immunity

carrier is cholera toxin B chain protein (CTB) further.
[0012]This invention provides more preferably a transgenic plant into which pBI121-CTB-JE was introduced.
[0013]This invention provides said transgenic plant in which said vegetation is tobacco.
[0014]This invention provides said transgenic plant for using it as Japanese encephalitis vaccine. . Explain this invention in detail below.
[0015]
[Embodiment of the Invention]The gene which encodes the fused protein of a membrane immunity carrier and Japanese encephalitis virus outline protein (JEV) is introduced into the transgenic plant of this invention. Said fused protein is the protein connected so that a membrane immunity carrier and Japanese encephalitis virus outline protein might serve as a yne frame.
[0016]Here, a "membrane immunity carrier" says membrane tissue the protein which has specific compatibility. Said membrane immunity carrier can use the protein which has compatibility, for example for bacteria, such as cholera toxin B chain protein, toxigenic Escherichia coli (LTB), a salmonella, or lactic acid bacteria, a virus, a pathogen, or the mucosal cell surface that originates in addition to this. In one mode of this invention, said membrane immunity carrier is cholera toxin B chain protein (CTB) preferably.
[0017]In one mode of this invention, said Japanese encephalitis virus outline protein is protein of a Japanese encephalitis virus, and is Eglycoprotein contained in an outline preferably.
[0018]Although the acquisition method in particular of the gene which encodes said fused protein is not limited, said DNA is producible as follows, for example.
[0019]Since the cDNA arrangement which encodes CTB and the Japanese encephalitis virus outline protein JEV (Eglycoprotein) is known, The gene of this invention can be obtained chemosynthesis or by making the DNA fragment which has this base sequence hybridize as a probe by designing a primer based on the arrangement and using the usual PCR method.
[0020]Said primer may include the arrangement which can unite E protein gene with the C' end side. A primer including the arrangement which enables fusion of a foreign gene with a yne frame may be designed to BamHI or the SpeI site of the Kozac arrangement for raising the expression efficiency in eukaryote to one end, and N'C' one end.
[0021]Specifically as a method of acquiring DNA which encodes cholera toxin B chain origin protein among said fused protein, it can amplify by performing PCR using the following reaction conditions, a template, and a primer, for example.;
Template: Plasmid pM2, N-terminal primer:5'-GCGCCATGGTTAAATTAAAATTTGGTGTT-3' (array number 1)
C-terminal primer:5 -- '-CGCGAGCTCTTAAAGTTCATCCTTTTCGGATCCTGGACTAGTAGGGGTACCGGGCCCGGGTCCATTTGC-3' (array number 2)
PCR conditions: In [in / on / in / on 94 ** and / after 3 minutes and / 94 ** / 45 seconds and 55 ** and / 1 minute and 72 **] 72 **, it is 10 minutes to 30 cycles and the next about 1 minute.
[0022]What is necessary is just to amplify Japanese encephalitis virus outline protein (Eglycoprotein) by the RT-PCR assay which made the RNA genome of a Japanese encephalitis virus the template.
Chemosynthesis of the designed primer can be carried out according to the base sequence. PCR can be performed in accordance with a conventional method. For example, it can amplify by performing PCR using

the following reaction conditions, a template, and a primer.;
N-terminal primer: 5'-GCGGGATCCACCTATGGCATGTGCACA-3' (array number 3)
C-terminal primer:(C1)
5'-GCGACTAGTTCCGAAGGGGGGTTCCAT-3' (array number 4)
(C2)
5'-GCGACTAGTAGCTTTATGCCAATGGTG-3' (array number 5)
(C3) 5'-GCGACTAGTCCCTTGTGTGATCCAAGA-3' (array number 6)
In [in / on / in / on 94 ** and / after 3 minutes and / 94 ** / 1 minute and 55 ** and / 1 minute and 72 **] 72 **, it is 10 minutes to 30 cycles and the next about 1 minute.
[0023]After a restriction enzyme cuts the amplified arrangement, for example, cloning of it can be carried out to a commercial plasmid. It is a publicly known method (for example) about this plasmid. J. Carry out isolation refining according to sambrook et al., Molecular Cloning, 2nd Ed., ColdSpring Harbour Laboratory Press, and pp.1.21-1.52. and publicly known methods, such as a Sanger's method and a Maxam-Gilbert method, -- and an automatic base-sequence-determination device (ABI DNA sequencer 310) uses, and a base sequence can be determined.
[0024]As long as it has immunity derivation activity, in the amino acid sequence concerned, variation, such as deletion, substitution, and addition, may produce said fused protein to at least one amino acid. For example, even if the above variation has few amino acid sequences of fused protein, one amino acid [about 1-50 / 1-20] may carry out deletion of them still more preferably preferably, Or 1-20 amino acid may add about 1-50 pieces to at least one amino acid sequence of fused protein still more preferably preferably, or the amino acid sequence of fused protein -- at least one piece may be preferably replaced by about 1-50 pieces and the still more desirable amino acid of others [amino acid / 1-20]. As long as it has immunity derivation activity, a longer peptide sequence may be encoded shorter. It is the Kunkel method in order to introduce variation into a gene. By the method according to a technique or this with the publicly known Gappedduplex method etc., it can carry out using the kit for variation introduction etc. which used the site-directed-mutagenesis method, for example.
[0025]Immunity derivation activity is activity which the animal medicated with said quality of fusion MPAKU recognizes said fused protein as a foreign matter, and produces the antibody to said fused protein inside of the body, and this antibody acts on the active part of said fused protein, and checks the activity of a Japanese encephalitis virus.
[0026]Next, how to produce the vector containing the gene which encodes the fused protein of a membrane immunity carrier and Japanese encephalitis virus outline protein (E glycoprotein) is explained.
[0027]Production of the recombinant vector for vegetable introduction and the recombinant vector for transformation vegetable introduction of Agrobacterium digest DNA obtained as mentioned above with a suitable restriction enzyme by remaining as it is or request, or can connect and build a suitable linker. Although middle vectors, such as pBI101, pBI121, the binary vector of pGA482 grade and pLGV23Neo, pNCAT, and pMON200, are mentioned as a vector for inserting DNA, it is not limited to these vectors.
[0028]the gene which encodes said fused protein needs to be included in a vector so that the function of the gene may be exhibited -- especially within a plant body, In order to make a foreign gene etc. reveal, it is necessary to arrange the promotor and terminator for vegetation before and after a structural gene, respectively. As an available promotor, they are 35S transfer object [Jefferson of cauliflower mosaic virus

(CaMV) origin, and R.A. et al. at this invention, for example. : The EMBO J 6:3901-3907(1987)], Ubiquitin [Christensen of corn, A.H. et al. :P lant Mol.Biol.18:675-689(1992)], They are mentioned by promotors, such as a nopaline synthesis enzyme (NOS) gene, an octopine (OCT) synthase gene, and an actin (Act1) gene of a rice, and as terminator arrangement, For example, the terminator of the cauliflower mosaic virus origin or nopaline synthesis enzyme gene origin, etc. are mentioned. However, if it is the promotor and terminator with which functioning within a plant body is known, it will not be limited to these things.

[0029]Intron arrangement with the function to reinforce gene expression between a promoter sequence and said DNA if needed, For example, the intron [Genes& Development1:1183-1200 (1987)] of the alcohol dehydrogenase (Adh1) of corn can be introduced. In order to choose the target transformed cell efficiently, an effective selection marker gene may be used together. The hygromycin phosphotransferase (htp) gene which gives the resistance over antibiotic hygromycin to vegetation as a selective marker used in that case, The phosphino SURISHIN acetyltransferase (bar) gene which gives the resistance over beer RAHOSU (bialaphos), the blasticidin S deaminase (BSD) gene which gives the resistance over blasticidin S, etc. are mentioned.

[0030]In order to minimize intermolecular friction of CTB and a fusion gene, a hinge region may be inserted between CTB and a fusion gene. The hinge region can make GPGP one unit and can build it three sorts to a single, double, and TORIPURU and a tandem, for example. In that case, by a vegetable species, the codon of a hinge region uses what has comparatively low frequency in use, and a ribosome should just halt or slow it down in the case of protein translation.

[0031]It rearranges within the endoplasmic reticulum of a plant cell, and protein is accumulated efficiently, and in order that fused protein may make a pentamer easy to form, an endoplasmic reticulum retention signal (SEKDEL) may be inserted in C' one end of fused protein. In that case, SEKDEL uses the highest thing of codon frequency and should just insert the stop codon TGA behind that.

[0032]By request, what contains cis- elements, such as an enhancer, a splicing signal, a poly A addition signal, a ribosome junction sequence (Shine Dalgarno sequence), etc. further can be connected.

[0033]In order to insert in a binary vector the gene which encodes fused protein, a suitable restriction enzyme cuts DNA refined first, and it is a suitable vector. The method of inserting in the restriction enzyme part or multi-cloning site of DNA, and carrying out ligation to a vector, etc. are adopted.

[0034]What is necessary is just to insert in pBI121 vector the gene which encodes said fused protein as an example of said recombinant vector, for example.

[0035]First, the expression vector containing the gene which encodes a CTB field downstream from a pBI system vegetable expression vector is built (drawing 3, 4). E protein region of JEV cDNA is inserted in such a vector, and the vector which can reveal JEV/CTB fused protein is built (drawing 5).

[0036]For example, the fusion gene containing the CTB gene built as mentioned above is set to $CTBh_{1-3}$SEKDEL, It inserts downstream from cauliflower mosaic virus 35S promotor in the plasmid vector pIBT210.1, and pIBT210.1-$CTBh_{1-3}$SEKDEL is produced (drawing 3).

[0037]Next, the whole T-DNA field which has a $CTBh_{1-3}$SEKDEL expression cassette from this plasmid vector is started by HindIII-EcoRI, It changes for the HindIII-EcoRI fragmentation of vegetable expression vector pBI121, and pBI121-$CTBh_{1-3}$SEKDEL is produced (drawing 4). If this vegetable expression vector has BamHI and a SpeI site downstream from CTB and a foreign gene is inserted in these restriction enzyme

sites, it becomes CTB and a yne frame and can produce CTB fused protein.
[0038]The overall length of JEVEglycoprotein is inserted in the BamHI-SpeI site of the above-mentioned pBI121-CTBh$_{1-3}$SEKDEL, and a CTB-JEVE fusion gene is built (drawing 5 (pBI121-CTB-JE)). DNA sequencer is used as mentioned above, the last base sequence of a fusion gene is checked, and it checks that CTB and a JEVE gene are yne frames.
[0039]Next, how to produce the transgenic plant which introduced the gene which encodes said fused protein is explained.
[0040]The vegetation as used in this invention means a vegetable cultured cell, the plant body of cultivated plants, vegetable organs (for example, a leaf, a petal, a stem, a root, a rhizome, a seed, etc.), or all of organizations (for example, epidermis, phloem, a parenchyma, xylem, a vascular bundle, etc.). As a vegetable kind used here, although there is no restriction in particular, various vegetation, such as a rice, wheat, tobacco, a tomato, and thale-cress, is mentioned. Preferably, they are solanaceous plants, such as a tomato, an eggplant, a green pepper, and tobacco. Tobacco is fried still more preferably.
[0041]As a gestalt of the vegetation into which a gene is introduced in this invention, the plant cell of all refreshable kinds of gestalt is contained in a plant body. For example, although a cultured cell, a protoplast, a shoot germ, many blastemas, a capillary root, and a callus are mentioned, it is not restricted to these. The cell in a plant body is also contained in the plant cell in this invention.
[0042]The transformation of vegetation with the gene which encodes said fused protein can be introduced into the engineer in this technical field using publicly known various methods. It can carry out by introducing into a vegetable host the vector containing said gene by the binary vector method, the party Kurgan method, or the polyethylene-glycol method of Agrobacterium, etc. Or it can also introduce and transform into a protoplast by the electroporation method.
[0043]The vector in which the transgenics by the party Kurgan method contains a selection marker gene directly, and the vector containing said gene can be mixed, and it can carry out by the cotransformation (co-transformation) method simultaneously fired into a vegetable cell. The shot obtained as a result of a transformation, a capillary root, etc. can be used for a cell culture, a tissue culture, or organ culture, and a plant body can be made to reproduce them by administration of the phytohormone of suitable concentration, etc. using the plant-tissue-culture method known conventionally.
[0044]When using the binary vector method of Agrobacterium, between the boundary arrangement (LB, RB) of the above-mentioned binary vector, an objective gene is inserted and this recombinant vector is amplified in Escherichia coli. Subsequently, the amplified recombinant vector is introduced into Agrobacterium tumefaciens LBA4404, EHA101, C58C1RifR, etc., and this is used for the transduction to vegetation.
[0045]For example, what is necessary is just to perform transduction as follows, when introducing into tobacco. The leaf of the non-recombination tobacco (N. tabacum) which carried out axenic culture is cut down, and it cuts in the size of the 1.5-2cm four quarters. The cut-off leaf attaches a crack to some places with a knife, and dips it in the culture medium (O. D_{600}=1x10) of Agrobacterium rearranged as mentioned above for 3 minutes so that infection may take place easily. Then, it removes as much as possible, and on MS agar medium, the Agrobacterium liquid attached to the leaf is certainly turned over, and it covers with it without a crevice (turning the stoma side up).
[0046]A petri dish is closed by parafilm, about two vents are opened, it incubates in a room temperature in a

dark room for three days, and infection is urged.
[0047]The leaf put into the dark room is moved to MS shootgrowth medium containing an antibiotic (kanamycin) (50 microg/(ml)) and a phytohormone (auxin and cytokinin) in three days, and a callus is derived. Since a bud begins to appear two to three weeks after a callus, move to Magneta Box of entering [which cuts out the bud which came out from a callus and contains only an antibiotic (kanamycin) (50 microg/(ml))] MS root growth medium, and vegetable axenic culture is produced, Recombination tobacco can be obtained.
[0048]The vegetation into which the gene of this invention was introduced can choose the transformed cell holding a gene in the analysis of the screening by a selective marker, a gene, or its expression product. For example, the check of the target gene being included in the obtained transgenic plant and its next generation, It can carry out by extracting DNA from these cells and tissue in accordance with a conventional method, and detecting the introduced gene using the publicly known PCR method or a Southern blot technique etc. The obtained transgenic plant is grown and slipped by the pot in which soil or a vermiculite was filled. Thus, the obtained transgenic plant is also contained in the range of this invention. The manifestation part within the plant tissue of the gene of this invention can be checked by analyzing the manifestation of mRNA in each organization, or a proteinic manifestation, for example. Although a RT-PCR assay, a Northern blot technique, etc. are mentioned and the Western analysis method using an antibody, etc. are specifically mentioned as the check method of the manifestation by this invention transgenic plant, it is not limited to these.
[0049]
[Example]Hereafter, an example explains this invention still more concretely. However, as for this invention, the technical scope is not limited to these examples.
[0050]1. Manifestation of CTB-JE by Agrobacterium.
[0051]The expression vector which contains the fused protein of cholera toxin B chain protein and Japanese encephalitis virus outline protein (Eglycoprotein) as is below construction of a method CTB-JEV E protein fusion gene was produced (drawing 3).
[0052]PCR amplification of the cholera toxin B chain gene was carried out from plasmid pM2 as a membrane immunological adjuvant, and it carried out as [unite / with the C' end side / E protein gene].When amplifying a CTB gene, N' one end introduces the Kozac arrangement (GCCATGG:ATG is an initiation codon) for raising the expression efficiency in eukaryote, and enabled it to unite a foreign gene with BamHI or the SpeI site of C' one end with a yne frame. PCR was performed using the following reaction conditions, the template, and the primer.
[0053]Template: Plasmid pM2, N-terminal primer:5'-GCGCCATGGTTAAATTAAAATTTGGTGTT-3' (array number 7)
C-terminal primer: 5'-CGCGAGCTCTTAAAGTTCATCCTTTTCGGATCCTGGACTAGTAGGGGTACCGGGCCCGGGTCCATTTGC-3' (array number 8)
PCR conditions: In [in / on / in / on 94 ** and / after 3 minutes and / 94 ** / 45 seconds and 55 ** and / 1 minute and 72 **] 72 **, it is 10 minutes to 30 cycles and the next about 1 minute.
[0054]In order to minimize intermolecular friction of CTB and a fusion gene, the hinge region (GPGP) was inserted between CTB and a fusion gene. The hinge region made GPGP one unit and built it three sorts to a

single, double, and TORIPURU and a tandem. At that time, by the vegetable species, the codon of a hinge region uses what has comparatively low frequency in use, and the ribosome halted or slowed it down at the time of protein translation. It rearranged within the endoplasmic reticulum of a plant cell, and protein was accumulated efficiently, and in order that fused protein might make a pentamer easy to form, the endoplasmic reticulum retention signal (SEKDEL) was inserted in C' one end of fused protein. SEKDEL used the highest thing of codon frequency and, specifically, inserted the stop codon TAA behind that (TCC GAA AAG GAT GAA CTT TAA : it is S E K E D E L, respectively it corresponds without a stop codon).

[0055]It inserted downstream from cauliflower mosaic virus 35S promotor in the plasmid vector pIBT210.1 by having set to $CTBh_{1-3}$SEKDEL the fusion gene built in this way, and pIBT210.1-$CTBh_{1-3}$SEKDEL was produced (drawing 3).

[0056]After digesting the insertion to pIBT210.1 by SacI (the SacI recognition site (GAGCTC) is included in said C terminal primer), it performed ligation by the usual method.

[0057]The overall length of $CTBh_{1-3}$SEKDEL was checked with an automatic base-sequence-determination device (ABI DNA sequencer 310). Next, the whole T-DNA field which has a $CTBh_{1-3}$SEKDEL expression cassette from this plasmid vector is started by HindIII-EcoRI, It changed for the HindIII-EcoRI fragmentation of vegetable expression vector pBI121, and pBI121-$CTBh_{1-3}$SEKDEL was produced (drawing 4). If this vegetable expression vector has BamHI and a SpeI site downstream from CTB and a foreign gene is inserted in these restriction enzyme sites, it becomes CTB and a yne frame and can produce CTB fused protein.

[0058]Next, the overall length of the Japanese encephalitis virus E glycoprotein gene was amplified by the RT-PCR assay from the RNA genome of a virus. It is about 30% (from the amino acid residue No. 300 of E glycoprotein.) by the side of 3' of a gene based on the cDNA. Insert in (C1) to the amino acid residue No. 377, insert in (C2) to the amino acid residue No. 399, and (C3) is inserted in a BamHI-SpeI site to the amino acid residue No. 426, The CTB-JEVE fusion gene was built (drawing 5 (pBI121-CTB-JE)), DNA sequencer was used as mentioned above, the last base sequence of the fusion gene was checked, and it checked that CTB and a JEVE gene were yne frames.

[0059]Here, produced pBI121-CTB-JE is explained with reference to drawing 3 - 5. The T-DNA field of pBI121-CTB-JE contains the following genes in the field inserted into RB and LB (when RB and LB are recognized with Agrobacterium and insert the copy of T-DNA into a vegetable core, they are an indispensable base sequence field).

[0060]1. The phosphotransferase gene (NPT-II) expression cassette (an NOS promotor and an NOS terminator field are included) which grants an antibiotic kanamycin resistant is included in a transgenic plant. This is a gene required for recombinant selection.

[0061]2. A CTB-JE expression cassette (eCaMV35S promotor *, TEV5'translation enhancer region ** and VSP3' terminator) is included. it be far from CTB protein to E protein by a hinge part by having inserted Japanese encephalitis virus E-glycoprotein the back of a hinge region, and ahead [of the SEKDEL endoplasmic reticulum retention signal] so that it might be set to a CTB-hinge region and in frame -- ******. The SEKDEL endoplasmic reticulum retention signal was combined immediately behind E-glycoprotein so that it might rearrange to a vegetable endoplasmic reticulum and protein might be accumulated. Leader peptide in N' end of CTB is an original thing of a CTB gene.

When shifting into the peri plasm of gram negative bacteria, it functions, but in the structure for a vegetable manifestation, the function which shifts to the endoplasmic reticulum in a plant cell is achieved.

[0062]* eCaMV35S promotor : a promotor's 5' side is a duplex (tandem), and original CaMV35 S twist also has high several time transfer efficiency.
[0063]* *TEV5' translation enhancer : it is arrangement of the virus origin infected with tobacco, and is a base sequence which raises a translation rate.
[0064]The binary vector (pBI121-CTB-JE) which has an introductory CTB-JEVE fusion gene of the pBI121-CTB-JE gene to Agrobacterium was introduced into Agrobacterium tumefaciens LBA4404 by the electroporation method. At the time of transgenics, the gene pulsar of Bio Rad was used and the same protocol as the transgenics to Escherichia coli performed. Specifically in 2.5 kV, 25 micro F, and 200 ohms, DNA of about 30 ng/mu l was performed using 1microl (DNA of about 30 ng(s)).
[0065]Agrobacterium by which transgenics was carried out was chosen by kanamycin (50 microg/(ml)), and the binary vector introduced by the plasmid miniprep method was taken out from Agrobacterium. It was checked by cutting with some restriction enzymes whether as compared with insertion before, there would be any change in the size.
[0066]Detection Agrobacterium of the fused protein originating in transformation Agrobacterium A YEB solution (rifampicin 50microg/ml), It cultivated by streptomycin 100mug/ml and 50mug/of kanamycin ml, and all the water soluble protein was extracted as follows using the bacteria protein extract (50 mMTris-Cl, 1mM EDTA, 100mM NaCl).
[0067]1. Add phenylmethylsulfonylfluoride (PMSF) of 8microper 1 g of transformation Agrobacterium l, and lysozyme (10mg/(ml)) of 80microl, and incubate at a room temperature for 20 minutes.
[0068]2. Add 4 mg [per 1 g of Agrobacterium] deoxychoic acid, and incubate at 37 **.
[0069]3. adding DNaceI (1mg/(ml)) per 1 g of Agrobacterium in the place lysate came to be alike of a place muddily -- the room temperature during about 30 minutes -- incubation.
[0070]4. Settle lysate by high-speed (about 40000g) centrifugality (30-minute heart at long intervals), and separate supernatant liquid.
[0071]Next, in order to check that the CTB-JE fused protein of transformation Agrobacterium origin is maintaining the specific compatibility over GM1 ganglioside which is a receptor of CTB, GM1-ELISA was performed in the following ways.
[0072]A microtiter plate 1. monosialoganglioside-GM1 (Sigma G-7641), In 4 **, it coats overnight by 100microl / well (3.0 microg/(ml) a GCC acid buffer (15mM Na2CO3, 35mM NaHCO3), PH9.6).
[0073]2. Wash a plate by PBST [phosphate buffered saline (PBS), 0.05% Tween-20], block in 37 ** for 2 hours by bovine serum albumin (BSA) 1%, and wash again.
[0074]3. Dilute a transformation Agrobacterium extract with PBS to several steps, and incubate in 4 ** overnight by 100microl / well. Then, washing.
[0075]4. Add the primary antibody (1000 to 5000 time dilution) which recognizes E protein of CTB or JEV, and incubate in 37 ** for 2 hours. Then, washing.
[0076]5. Add the second antibody (100microl / well) of alkaline-phosphatase conjugate, and incubate in 37 ** for 1 hour. Then, washing.
[0077]6. Measure by O.D.415nm after adding an alkaline-phosphatase substrate (100microl / well) and

making it color for 20 minutes in a room temperature.
[0078]7. Use CTB protein (Sigma) as control and measure the CTB fused protein of vegetable origin.
[0079]It was checked that result transformation Agrobacterium is producing the CTB-JEVE fused protein which exists in the lower stream by cauliflower mosaic virus 35S promotor.
[0080]2. Mouse membrane immunity experiment which uses fused protein of Agrobacterium origin.
[0081]The immunity experiment of a mouse was conducted using the crude extract of a method biomass. As an immunity route, 1 time of four total immunity per week was carried out and carried out from three routes of taking orally, pernasality, and the abdominal cavity. Antibody titer was measured using the blood serum on which it voted from the tail one week after the last immunity. In the protection test, using the Beijing stock of a Japanese encephalitis virus, the case where the number of plaques was halved in a 10 or more-time blood serum degree of dilution was made into the neutralizing antibody positivity as carried out by the Japanese encephalitis vaccine examination.
[0082]The derivation of the antibody with which the antibody to a cell component does not go up, but only the route by result taking orally and pernasality recognizes only expression protein to be specifically was checked. By the internal use group, it turned out that four animals are producing the virus neutralizing antibody among five animals.
[0083]By mucosal administration, the fused protein can acquire an equivalent effect, although the conventional Japanese encephalitis vaccine prescribing [for the patient]-a medicine method performs antibody derivation by injection inoculation. That is, it is possible by producing fused protein with cholera toxin B chain protein to carry virus outline protein in intestinal-mucosa tissue efficiently, and to derive a systemic immune response (Table 1).
[0084]
[Table 1]

接種ルート	ELISA No.positive/mice (OD)		中和抗体
	CTB-JE	菌体成分	
経口	5/5 (0.465)	0/5 (0.024)	4/5
経鼻	5/5 (0.486)	0/5 (0.069)	1/5
腹腔	5/5 (2.430)	5/5 (0.576)	1/5

3. Derivation experiment of virus neutralizing antibody by rhinovaccination of Japanese encephalitis vaccine.
[0085]In order to investigate the antibody induction potency by the membrane immunity of a method present Japanese encephalitis virus vaccine, the pernasal immunity experiment was conducted using the mouse. It inoculated a total of 4 times once per week, and antibody titer was measured one week after the last immunity. About the protection test, it carried out like the case of the above-mentioned CTB-JE fused protein ingestion.
[0086]In order to investigate the efficiency of a membrane immunity route, after diluting and carrying out pernasal immunity of the present Japanese encephalitis vaccine, antibody titer and neutralizing antibody value were investigated by ELISA.
[0087]A result result is as in Table 2. The rise of antibody titer was accepted after three weeks of immunity starts. Although the rise of antibody titer was accepted also by mixing with a vaccine independent or an

adjuvant, the remarkable rise was checked by ELISA antibody titer and neutralizing antibody value by mixing of an adjuvant (Table 2).

[0088]

[Table 2]

接種ルート	抗原（ng）	ELISA No.positive/mice (OD)	中和抗体価
経鼻	日本脳炎ワクチン（384ng）	3/5 (0.198)	5/5 (624)
経鼻	日本脳炎・CT 混合ワクチン（384ng）	5/5 (1.123)	5/5 (2,154)

[0089]The efficiency of the membrane immunity route became clear [that triple the amount / about / of a vaccine is required also of the case of a mixed vaccine] as compared with the amount of vaccines (134ng) used for abdominal administration. When an adjuvant was not used, even if it prescribed the 10 times the amount for the patient, the rise of antibody titer was not checked (Table 3).

[0090]

[Table 3]

	日本脳炎ワクチン				
ワクチン量（ng）	1,200	400	134*	45	15
ELISA	0/4	0/4	0/4	0/4	0/4
中和試験	0/4	0/4	0/4	0/4	0/4

日本脳炎ワクチン＋CT				
1,200	400	134*	45	15
4/4	3/4	0/4	0/4	0/4
4/4	4/4	1/4	1/4	0/4

*134 ng は通常のマウス実験でワクチンに使用される量

4. Manifestation of fused protein by transgenic tobacco.

[0091]It inserts in Agrobacterium (Agrobacterium tumefaciens) which is a kind of soil bacteria with the capability to introduce into the vegetable nuclear staining inside of the body binary vector pBI121-CTB-JE in which the tobacco by method Agrobacterium infection carried out transformation construction. Agrobacterium which has pBI121-CTBh$_{1\text{-}3}$SEKDEL in a biomass Rifampicin (50 ng/ml), the YEB culture medium (perl. -- : -- Beef Extract -- 5 g) containing streptomycin (100 microg/(ml)) and kanamycin (50 microg/(ml)) Bacto yeast extract 1g, Sucrose 5g, MSO culture medium which cultivates for three days by Bacto Ager 9g and pH 7.3, and contains acetosyringone (370microM) and which sterilized (1 pack/l MS salts) It was suspended so that it might be set to 109 to 1010 bacteria/about ml 3.0% sucrose, 1xB5 vitamins, and pH 5.8. After cultivating to the extent that the meaning of the tobacco which sterilized was budded by the MS culture medium and seven to eight leaves came out, the leaf was taken and it started in the shape of a lattice with the scalpel (about 2 cm around). These end ****** leaf disk attaches a crack to some places with a knife so that infection may take place easily, It dipped in the suspension of transformation Agrobacterium for 1 to 2 minutes, after taking

out, the excessive fluid was removed, and on MS agar medium, it covered without the crevice so that the back side might certainly turn up (turning the stoma side up). The petri dish was closed by parafilm, about two vents were opened, it incubated in the room temperature in the dark room for three days, and infection was urged. Three days afterward the leaf disk was removed and it moved to MS selective medium containing kanamycin (50 microg/(ml)), NAA (100 microg/(ml)), and claforan (300 microg/(ml)). After callus-ization advanced by ** and others [about two-week], the shot was formed, and when the leaf became about 2-3 sheets, it started from the callus, and culture was individually continued by MS selective medium. After a root was formed from a shot and a plant body begins to have grown, it transplanted to the ground and was made to grow up further.

[0092]Genomic DNA is extracted from the tobacco in which the analysis kanamycin resistant of transformation tobacco is shown, PCR amplification (the beginning -- 94 -- ** -- three -- a minute -- the next -- 94 -- ** -- 45 -- a second -- 55 -- ** -- one -- a minute -- 72 -- ** -- one -- a minute -- 30 -- cycles -- the last -- 72 -- ** -- 10 minutes) was performed using the primer set and arrangement which amplify a CTB field specifically, and it checked that tobacco was transformed. The tobacco transformed is a vegetable protein extraction buffer (200 mM Tris-Cl) at the following points. pH 8.0 and 100 mM NaCl, 400mM Sucrose, All the water-soluble protein was extracted using 10mM EDTA, 14mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.05% Tween-20.

[0093]1. per [the plant tissue (that of tobacco is a rhizome of a potato, etc.) of one g] -- carry out with the earthenware mortar of Hikami using a 1-ml extraction buffer.

[0094]2. It is the 15-minute heart at long intervals at 17000xg and 4 ** about homogenate.

[0095]3. Collect supernatant liquid and it is centrifugality once again.

[0096]4. Use supernatant liquid for protein assay. Protein is set to 10-20microl per about 50-100microg at this time.

[0097]The protein extract was used, and in order to check that CTB-JE fused protein is maintaining the specific compatibility over GM1 ganglioside which is a receptor of CTB, GM1-ELISA was performed in the following ways.

[0098]A microtiter plate 1. monosialoganglioside-GM1 (Sigma G-7641), By 100microl / well (3.0 microg/(ml) a GCC acid buffer (15mM Na2CO3, 35mM NaHCO3), PH9.6), it coats in 4 ** overnight.

[0099]2. Wash a plate by PBST [phosphate buffered saline (PBS), 0.05% Tween-20], block in 37 ** for 2 hours by bovine serum albumin (BSA) 1%, and wash again.

[0100]3. Dilute a transformation Agrobacterium extract with PBS to several steps, and incubate in 4 ** overnight by 100microl / well. Then, washing.

[0101]4. Add the primary antibody (1000 to 5000 time dilution) which recognizes E protein of CTB or JEV, and incubate in 37 ** for 2 hours. Then, washing.

[0102]5. Add the second antibody (100microl / well) of alkaline-phosphatase conjugate, and incubate in 37 ** for 1 hour. Then, washing.

[0103]6. Measure by O.D.415nm after adding an alkaline-phosphatase substrate (100microl / well) and making it color for 20 minutes in a room temperature.

[0104]7. Use CTB protein (Sigma) as control and measure the CTB fused protein of vegetable origin.

[0105]As a result of conducting number solids analysis of the result transgenic tobacco, it was checked that the recombination CTB-JE fused protein of per [originating in the leaf of tobacco / all the water-soluble-

protein 1g] and 2-12microg is produced (drawing 6).
[0106]
[Effect of the Invention]Although the conventional Japanese encephalitis vaccine prescribing [for the patient]-a medicine method performs antibody derivation by injection inoculation, it can acquire an effect equivalent to it by the mucosal administration of fused protein in this invention. That is, it is possible by producing fused protein with cholera toxin B chain protein to carry virus outline protein in intestinal-mucosa tissue efficiently, and to derive the immune response of a whole body system. Since creation of the transgenic plant into which this fused protein was introduced is possible. By carrying out gene expression to edible vegetation, it is possible to carry out immunity via a direct intestinal mucosa. The above-mentioned composition and its membrane immunity prescribing [for the patient]-a medicine method can be applied to a vertebrate at large [containing Homo sapiens and livestock animals], and the use as "a vaccine to eat" is expected. [0107]
[Layout Table]
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[Translation done.]